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## (57) Abstract

DNA molecules which encode a 255 amino acid polypeptide, comprising the 32 amino acid M-CSF leader sequence and the 223 amino acid mature M-CSF polypeptide, and lack the coding sequence for the carboxy-terminal propeptide sequence present in wild-type M-CSF DNA, result in highly efficient production of M-CSF, which displays similar post-translational modifications as wild-type M-CSF and is unaffected in biological properties.

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## PROCESS FOR PRODUCING M-CSF 223

## FIELD OF THE INVENTION

5 The present invention relates to improved processes for producing macrophage-colony stimulating factor (M-CSF or MCSF). More specifically, this invention relates to a method employing a novel DNA sequence encoding directly for a 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader and the 223 amino acid mature M-CSF polypeptide, and a recombinant DNA molecule encoding a 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader and the 223 amino acid mature M-CSF polypeptide.

## BACKGROUND

15 Colony-stimulating factors are known to be required for the proliferation and differentiation of hematopoietic cells, such as erythrocytes, granulocytes, macrophages, eosinophils, platelets and lymphocytes. MCSF is specific to the formation of macrophages. It is expected that M-CSF would be useful in the treatment of leukocytopenia resulting from chemotherapy or leukocyte levels after bone marrow transplantation. Wild-type M-CSF is expressed in Chinese hamster ovary (CHO) cells as a 522 amino acid membrane anchored precursor protein which is proteolytically processed at amino acid 223 to generate the 45 kDa amino terminal-derived soluble mature form (Figure 1). M-CSF for therapeutic use has been purified as a 90 kDa homodimer whose carboxy-terminus is amino acid 223. In addition, CHO cells expressing the wild-type gene secrete significant amounts of a heterogenous high molecular weight species whose carboxy-terminal sequences extend beyond the residue 223 cleavage site, resulting in high molecular weight heterogeneity of the recombinant protein. Within these extended sequences, glycosaminoglycan addition occurs which contributes to high molecular weight heterogeneity.

20 The production of human M-CSF (rhM-CSF) by recombinant means has been accomplished using DNA sequences encoding a polypeptide of 554 amino acids. Clark et al., United States Patents 4,868,119 and 4,879,227. This polypeptide is cleaved by mammalian cells to form the 223 amino acid mature M-CSF polypeptide.

25 Takaku et al., European Patent application (EP) 0 276 551 and United States Patent 5,114,710 disclose the use of 189, 214 and 238 amino acid polypeptide sequences encoding M-CSF to treat thrombocytopenia. However, Takaku et al. do not disclose or suggest the preparation of a 223 amino acid mature M-CSF polypeptide, nor do they disclose

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recombinant means or DNA nucleotide sequences encoding a 223 amino acid mature M-CSF molecule. Rather, Takaku et al. disclose methods of purifying M-CSF from human urine. See, EP 0 276 551 at pages 7 to 18.

5 Kawashima et al., EP 0385 385 discloses that the subunit protein of the homodimer of rhM-CSF has 223 amino acid residues. However, Kawashima et al. disclose methods of preparing 238 amino acid M-CSF polypeptides purified from human urine and does not disclose a nucleotide sequence encoding a 223 amino acid M-CSF polypeptide.

Toyo Jozo Co., UK Patent Application (GB) 2 249 100 A discloses a recombinant DNA vector capable of expressing a 179 amino acid M-CSF polypeptide.

10 Kawasaki et al., Science, 230:291-296 (1985) disclose a recombinant DNA sequence encoding a 256 amino acid M-CSF polypeptide, which includes a 32 amino acid leader sequence. This 224 amino acid mature protein (after removal of the leader sequence) is structurally distinct from the 223 amino acid form disclosed here in that amino acids 150 through 224 are different from amino acids 150 to 223 of the M-CSF polypeptide of the  
15 present invention.

### SUMMARY OF THE INVENTION

To increase the productivity of cell lines for the 90 kDa homodimer, we have developed an expression vector designated pMemcMAT which is designed to produce only  
20 the 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader and the 223 amino acid mature form of M-CSF (Figure 3). Surprisingly, the 255 amino acid form of M-CSF is properly processed when expressed in CHO cells, despite the fact that the polypeptide is not synthesized as part of a membrane-bound precursor protein due to the deletion of the carboxy-terminal sequences. Importantly, such cell lines do not produce the high molecular  
25 weight proteoglycan form of M-CSF.

Accordingly, it is one object of the present invention to provide recombinant DNA vectors capable of directly expressing a 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader and the 223 amino acid mature M-CSF polypeptide.

It is another object of the present invention to provide improved mammalian cell lines capable of directly expressing a 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader and the 223 amino acid mature M-CSF polypeptide.

It is yet another object of the present invention to provide improved processes for the production of M-CSF by culturing transformed mammalian cell lines which are capable of directly expressing a 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader and the 223 amino acid mature M-CSF polypeptide.

It is one feature of the present invention that cell lines transformed with DNA directly encoding a 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader and the 223 amino acid mature M-CSF polypeptide are used.

It is one advantage of the present invention that M-CSF is produced with improved yield and process efficiency.

It is another advantage of the present invention that the M-CSF produced is post-translationally modified (e.g., N-linked and O-linked glycoylation) similarly to rhM-CSF expressed from the full-length cDNA sequence.

It is another advantage of the present invention that the high molecular weight heterogeneity normally occurring with the production of rhM-CSF in wild-type cell lines is largely or wholly eliminated.

Other objects, features and advantages of the present invention will become more readily apparent from the following description.

### BRIEF DESCRIPTION OF THE FIGURES

**Figure 1:** The amino acid sequence of wild type M-CSF is shown. Asterisks and arrows (^) indicate the position of the inserted termination codons and EcoRI digestion site, respectively. The potential chondroitin sulfate addition site in the propeptide region is underlined.

**Figure 2:** Expression vector pMemc for wild-type M-CSF. pMemc contains the dihydrofolate reductase (DHFR) gene, which confers resistance to methotrexate. Transcription of a dicistronic mRNA is directed by the adenovirus major late promoter (MLP). The 5' end of the mRNA contains the adenovirus tripartite leader sequence (TPL).

5 followed by the full length M-CSF coding sequence (MCSF). Translation of the 3' proximal selectable marker gene is under control of the Encephalomyelocarditis virus leader sequence (EMC-L). The vector also contains the SV40 origin of replication and enhancer (SV40 ori), adenovirus VAI gene (VA) and the SV40 polyadenylation site (SV40-pA). Selected restriction digestion sites are indicated.

10 **Figure 3:** Expression vector for MCSF-223. Termination codons and an EcoRI restriction site were inserted immediately following the coding sequences for the carboxy terminus of the 45 kDa monomer at amino acid residue 223 by site-directed DNA mediated mutagenesis of pMemc. EcoRI digestion was performed to delete the propeptide coding sequences to generate pMemcMAT. The same regulatory elements are present on pMemcMAT as described for pMemc in Figure 2.

### DESCRIPTION OF THE SEQUENCES

15 SEQUENCE ID NO. 1 is the nucleotide sequence encoding the 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader and the 223 amino acid mature M-CSF polypeptide.

SEQUENCE ID NO. 2 is the amino acid sequence of the 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader and the MCSF-223 polypeptide.

20 SEQUENCE ID NO. 3 is the amino acid sequence of the 186 amino acid polypeptide comprising the DHFR coding sequence which confers resistance to methotrexate and may be used as a selectable marker.

SEQUENCE ID NO. 4 is the amino acid sequence of the wild-type M-CSF polypeptide, including the leader sequence and 522 amino acid.

### 25 DETAILED DESCRIPTION OF THE INVENTION

In order to accomplish the above objectives, the inventors followed a program first of generating new cell lines. This program comprised construction of vectors for the production of the 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader and the 223 amino acid mature M-CSF polypeptide; selection, amplification and cloning of

transformed cells; and measuring the productivity of the cells thus obtained. The initial cell lines and proteins produced thereby were then characterized.

Second, high productivity cell lines were characterized and adapted to cell culture conditions. Next, cellular and volumetric productivity were measured, followed by identification of growth characteristics.

Third, the 223 amino acid mature M-CSF protein, as directly expressed by the DNA sequence encoding the 255 amino acid polypeptide, comprising the 32 amino acid M-CSF leader and the 223 amino acid mature M-CSF polypeptide, was characterized and compared to the protein produced by expression of the full length (wild type) M-CSF polypeptide following intracellular proteolytic processing. N-terminal and C-terminal amino acid sequences were analyzed. The protein was analyzed by Achrobacter protease K peptide mapping. The N-glycan fingerprint was determined. Chromatographic analyses, including reversed phase and size exclusion HPLC, were performed. Electrophoretic analyses, including polyacrylamide gel electrophoresis and isoelectric focusing, were performed.

Fourth, the biological activity of the M-CSF produced by the DNA sequences of the present invention was measured and compared to that of M-CSF produced using wild type cell lines. In vitro activity was measured in the 32Dcfms assay and murine bone marrow assay. Half-life, clearance rate, and volume of distribution of M-CSF polypeptide in rats was measured. Finally, incidence of monocytosis and thrombocytopenia in the rat was observed.

Accordingly, the present invention comprises isolated and purified DNA molecules which consist essentially of the DNA sequence of Sequence ID No. 1. The DNA molecules of the present invention encode the production of a 255 amino acid polypeptide, comprising the 32 amino acid M-CSF leader and a 223 amino acid polypeptide having M-CSF activity and appropriate post-translational modifications, said polypeptide lacking the carboxy-terminal propeptide sequence, including a transmembrane domain, present in wild-type recombinant M-CSF precursor polypeptide before intracellular processing when produced by

mammalian cells. In a preferred embodiment, the DNA molecule of the present invention comprises the vector pMemcMAT.

5 The present invention also comprises isolated and purified DNA molecules consisting essentially of a DNA sequence encoding the polypeptide comprised of the amino acid sequence of Sequence ID No. 2. Because of the degeneracy of the genetic code, it will be recognized that numerous changes to the DNA sequence of Sequence ID No. 1 can be made without altering the amino acid sequence of the polypeptide encoded by said DNA sequence. See, for example, Lehninger, Biochemistry, 2d ed., pp.959-963 (Worth Publishers, New York 1975). The DNA molecules of the present invention encode the production of a 255  
10 amino acid polypeptide comprising the 32 amino acid M-CSF leader sequence and a 223 amino acid polypeptide having M-CSF activity, said polypeptide lacking the carboxy-terminal propeptide sequence, including a transmembrane domain present in wild-type recombinant M-CSF precursor polypeptide before intracellular processing when produced by mammalian cells.

15 The present invention also comprises methods of producing MCSF-223 protein comprising culturing in a suitable culture medium cells transformed with a DNA molecule comprising the DNA sequence of Sequence ID No. 1 and isolating and purifying said protein from said culture medium. In a preferred embodiment, the method of the present invention comprises culturing in a suitable culture medium cells transformed with pMemcMAT and  
20 isolating and purifying said protein from said culture medium. Suitable culture media are known in the art and depend upon the species of the host cells to be used to produce the MCSF-223 protein. Where the host cells to be used are Chinese Hamster Ovary (CHO)

cells, for example, suitable culture media include those described in Hamilton and Ham, In Vitro, 13: 537-547 (1977) and Mendiaz et al., In Vitro Cellular and Developmental Biology, 22: 66-74 (1986).

In another embodiment, the present invention comprises mammalian cell lines capable of producing MCSF-223 protein, said mammalian cell line comprising a DNA sequence directly encoding a 255 amino acid polypeptide comprising the 32 amino acid M-CSF leader sequence and the 223 amino acid mature M-CSF polypeptide, said polypeptide lacking the carboxy-terminal propeptide sequence present in wild-type recombinant M-CSF precursor polypeptide before intracellular processing when produced by mammalian cells.

The present invention further comprises other improvements to the process for producing M-CSF polypeptide. These improvements include the use of ultrafiltration/diafiltration steps, the use of alternate resins, and the development of the manufacturing process.

## Definitions

As used in the present application, the term "M-CSF activity" refers to at least one of the "CSF-1-like biological properties" as defined in United States Patent 4,868,119, the text of which is hereby incorporated herein by reference.

As used in the present application, the term "MCSF-223", "MCSF-223 protein" or "MCSF-223 polypeptide" refers to an amino acid sequence with M-CSF activity, which is 223 amino acids in length, but which is free of the carboxy-terminal propeptide sequence normally present in wild-type M-CSF before intracellular processing when produced by

mammalian cells. The MCSF-223 protein of the present invention is produced through recombinant means from MCSF-223 DNA, without the carboxy-terminal propeptide.

As used in the present application, the terms "MCSF-223 vector" and "MCSF-223 DNA vector" refer to recombinant DNA vectors capable of directly expressing a 223 amino acid mature M-CSF polypeptide. Such an MCSF-223 vector will comprise a coding DNA sequence of approximately 765 nucleotides in length, such that it will encode the 223 amino acid mature M-CSF polypeptide and include DNA encoding the 32 amino acid leader peptide which is considered to encompass amino acids -32 to -1 of Figure 1. It will be understood in the art, such vectors may further comprise DNA sequences not directly encoding the M-CSF mature protein, such as regulatory elements, e.g., promoters, leaders, or enhancers, and/or DNA sequences unrelated to M-CSF, e.g., signal peptides and selectable marker genes. However, the MCSF-223 vectors are free from DNA encoding the carboxy-terminal propeptide normally present in wild-type M-CSF precursor protein.

As used in the present application, the term "MCSF-223 cell line" refers to mammalian cell lines capable of directly expressing an MCSF-223 polypeptide. The MCSF-223 cell lines of the present invention are transformed with MCSF-223 vector DNA, so that they directly encode the production of MCSF-223.

As used in the present application, the terms "wild-type M-CSF", "wild-type M-CSF cell line" and "wild type M-CSF DNA vector" refer to DNA, vectors and cell lines which produce an M-CSF polypeptide significantly longer than 223 amino acids in length. The wild-type M-CSF precursor protein is 522 amino acids in length and contains a carboxy-terminal propeptide sequence which is normally cleaved during intracellular processing by

mammalian cells to generate the mature form of 223 amino acids. However, a significant proportion is cleaved at sites beyond 223 to generate high molecular weight M-CSF proteoglycan species.

### Examples

#### **Example 1. Construction of M-CSF expression vectors.**

A 1790 bp human M-CSF cDNA fragment derived from the M-CSF expression plasmid p3AMCSF-R1 was inserted into the expression vector pED to generate the vector pMemc. (Figure 1). pMemc contains the dihydrofolate reductase (DHFR) gene which confers resistance to methotrexate (MTX). Transcription to generate a dicistronic mRNA is directed by the adenovirus major late promoter in combination with the SV40 enhancer. The 5' end of the mRNA encodes M-CSF while the 3' end encodes the selectable and amplifiable marker DHFR. Translation of the 3' proximal gene is under the control of the encephalomyelocarditis virus (EMCV) leader sequence. The presence of this sequence between the M-CSF coding sequence and the selectable marker allows efficient internal entry of ribosomes to initiate protein synthesis of the selectable marker.

To increase the productivity of cell lines for the 90 kDa homodimer, we have developed an expression vector designated pMemcMAT to produce only the 255 amino acid polypeptide, comprising the 32 amino acid M-CSF and the mature 223 amino acid form of M-CSF (Figure 2). This was accomplished by site directed DNA mediated mutagenesis of pMemc. Protein synthesis termination codons and an EcoRI restriction site were inserted immediately following the coding sequence for amino acid residue 223. EcoRI digestion of the vector resulted in removal of the 3' terminal sequences encoding the extended carboxy-

terminal propeptide region. Removal of these sequences precludes potential readthrough translation of the termination codons to generate extended protein products. pMemcMAT directs expression of the mature form of M-CSF of 223 amino acid residues and confers resistance to MTX. pMemcMAT was deposited with the ATCC on December 11, 1992 and has been accorded ATCC accession number 75378. **Example 2. Development of CHO cell lines expressing M-CSF.**

**A. Development and characterization of MCSF-223 producing cell lines.**

pMemcMAT was introduced into CHO DUKX cells by lipofection and cells were subsequently selected for growth in the absence of nucleosides and the presence of 0.02 uM or 0.1 uM MTX. MTX resistant cells were picked into 16 independent pools and subjected to further rounds of selection and amplification in increasing concentrations of MTX. M-CSF production was monitored primarily using an ELISA assay developed at Genetics Institute which detects total M-CSF antigen. The highest producing cells were identified and subcloned by limiting dilution to produce clonal cell lines for further development. (Table I). The cellular productivity of these cell lines was quantitated by both ELISA assay and size exclusion chromatography (SEC) to monitor the levels of total M-CSF antigen and 90 kDa dimer, respectively. Cellular productivity ranged from about 30 to about 70 pg/cell/24h of total secreted antigen for individual cell lines. This represents an approximate 10-25 fold increase in productivity compared to previous production using wild type expressing cell line. The current wild type cell line produces about 3-5 pg/cell/24h under similar assay conditions.

TABLE I

MCSF-223 CHO Cell Lines			
Cell line	MTX (uM)	Total antigen pg/cell/24hr	90 kDa dimer pg/cell/24hr
Line H1	2	30	28
Line H8	5	40	42
Line H1L	5	70	ND
Line C10	5	30	20
Line D2	2	50	40
Line D1	5	40	ND

**Legend:** MCSF-223 cell lines were fluid changed into complete alpha medium (total antigen) or alpha defined medium without BSA (90 kDa dimer). Conditioned medium was harvested and cell numbers determined 24 h later. Cellular productivity was calculated by dividing volumetric productivity by cell number. MCSF total antigen levels were determined by ELISA. Levels of 90 kDa dimer were determined by size exclusion chromatography (SEC). Both values are given in pg/cell/24h. MTX, methotrexate; ND, not determined.

Recent reports have shown that the high molecular weight heterogenous M-CSF species contains a proteoglycan with carboxy terminal sequences which extend beyond the 223 cleavage site. Two potential glycosaminoglycan sites are carboxy-terminal to the cleavage site at amino acid 223 (see Figure 1). Thus, expression of the MCSF-223 polypeptide form should result in the absence of the high molecular weight proteoglycan species since the carboxy-terminal sequences including the glycosaminoglycan site are absent.

Analysis of unlabeled conditioned medium by SEC confirms lesser quantities of high molecular weight material secreted from MCSF-223 producing cells compared to wild type M-CSF. Two distinct peaks of high molecular weight material were detected by SEC of medium conditioned by MCSF-223 producing cells. Western blot analysis indicated that the material in one peak is M-CSF, which upon reduction has the electrophoretic mobility of 45 kDa monomer, while the other HMW peak is CHO cell specific protein. This HMW material is presumably different from that HMW material present in the wild-type cell lines. Approximately 89% of the material retained between 10 and 15 minutes consists of 90 kDa homodimer. In sharp contrast, SEC analysis of medium conditioned by wild type M-CSF producing cells detected a much higher proportion of high molecular weight species. This high molecular weight species does not display an electrophoretic mobility of 45 kDa following reduction, indicating it is different from the small amount of high molecular weight material observed for MCSF-223. This represents a significant advantage of the MCSF-223 cell lines, from both a yield and protein purification perspective, over wild-type M-CSF cell lines.

### **Example 3. Secretion of M-CSF in Bioreactors.**

Cell line H1 (Table I) was adapted to serum-free suspension culture. In petri dishes, 90 kDa M-CSF was produced by the resulting cell line at 14.7 pg/cell/day, whereas 90 kDa M-CSF was produced by a wild-type M-CSF cell line at only 2.0 pg/cell/day. The adapted H1 cell line was also grown in a 250-L bioreactor. In a series of seven 3-day or 4-day batch growth cycles, 90 kDa M-CSF was produced at 10.2 pg/cell/day, whereas in a series of 22

3-day batch growth cycles, 90 kDa M-CSF was produced by the wild type cell line at only 1.6 pg/cell/day. Mean cell densities were as high or higher during the cycles using the H1 cell line than during the cycles using the wild type cell line. Thus, the improvement in cellular productivity (pg/cell/day) leads to an improvement in overall productivity (g/day) cell line H1 has been deposited with the ATCC and has been accorded ATCC designation CRL 11275.

#### Example 4. Protein Characterization.

Critical to the development of an MCSF-223 producing cell line was comparison of the MCSF-223 to the wild type M-CSF material. This characterization process required purification of M-CSF produced from various MCSF-223-producing cell lines and from wild type cell lines. Material from cells grown in roller bottles was purified by DEAE/Q-Sepharose tandem chromatography followed by Jacalin agarose affinity chromatography. Final purification was done using Sephacryl S-300 chromatography. This purification process confirmed that MCSF-223 cell lines secreted predominantly the 90 kDa dimer species of M-CSF with little high molecular weight material (< 10%) and no propeptide in the conditioned medium. High molecular weight M-CSF and propeptide are the major contaminants of the wild-type M-CSF process. This indicates that purification of MCSF-223 is preferable to wild type M-CSF. Purified material was then characterized by a variety of methods as outlined below.

Amino-terminal and carboxy-terminal sequence analysis of MCSF-223 from a single cell line indicated that the predominant amino-terminal amino acid sequence corresponded

to the mature terminus beginning with EEVSE and that the predominant carboxy-terminal amino acid sequence corresponded to the correct mature terminus of RPPR. This important result indicates that termination of the protein at amino acid residue 223 does not result in aberrant proteolytic processing to generate truncated carboxy-terminal species.

5           Peptide mapping of *Achrobacter* protease K digested MCSF-223 from line H1 compared to wild type M-CSF from current wild type cell line showed similar but not identical profiles. All peptides present in the MCSF-223 chromatogram were also present in the wild type chromatogram and no peptides were absent inclusive of both the amino-terminus and carboxy-terminus peptides. Since the amino-terminus and carboxy-terminus of  
10           MCSF-223 correspond to those of wild type based upon amino acid sequence analysis and peptide maps were similar, no major structural differences appear to exist between MCSF-223 and wild-type M-CSF protein. It was observed that MCSF-223 displayed a distribution of peptides K12K13 and K14 different from that of the wild-type M-CSF. These peptides from wild-type M-CSF are known to reflect variability in the amount of O-linked  
15           glycosylation and sialation. The profile from MCSF-223 shows increased amounts of presumably more highly O-glycosylated and/or sialated peptides which are naturally present at lower levels in wild-type M-CSF. This analysis suggests that MCSF-223 contains increased but not abnormal O-linked glycosylation and/or sialation compared to wild-type M-CSF.

20           Comparative analysis of N-linked glycosylation of MCSF-223 from several cell lines compared to wild-type M-CSF from M $\alpha$ 3-18 cell line was performed using high pH anion exchange chromatography (HPAEC) of the N-glycans liberated from the protein by PNGase

digestion. Chromatograms of a representative MCSF-223 polypeptide derived from MCSF-223 line H1 cells and wild-type M-CSF derived from current wild type cells demonstrate that the spectrum of N-linked glycans is quite similar, but not identical, between MCSF-223 and wild-type forms of M-CSF. Specifically, there exist two areas of the N-linked fingerprint which display slight differences between MCSF-223 and wild-type M-CSF. MCSF-223 exhibits lower levels of monosialyted species eluting at approximately 20 minutes in the profile. Slight variation in peak ratios exists in the trisialyted species eluting at approximately 40 minutes in the profile. The exact structural basis for these differences is not known, but it is believed that differences in terminal sialylation are responsible. Preliminary data obtained by Warren assay to determine sialic acid content indicates that MCSF-223 contains more sialic acid than wild-type. These characterization studies indicate that carbohydrate addition and processing of MCSF-223 is not significantly altered compared to wild-type. MCSF-223 displays increased O-linked glycosylation and sialation. However, this appears to be the result of increased levels of carbohydrate moieties naturally present in the wild type M-CSF population rather than the appearance of species unique to MCSF-223.

Analysis of MCSF-223 by SEC indicated that the amounts of high molecular weight (HMW) species present in conditioned medium was, as expected, lower than in wild type M-CSF producing cells. However, a small amount of high molecular weight material was present in the medium. The reduction of the HMW species produced by the MCSF-223 cell lines to 45 kDa MW differentiates it from the HMW species produced by wild-type cell lines. Wild type HMW material upon reduction exhibits a mw of 150-200 kDa due to the presence of the glycosaminoglycan and the extended carboxy-terminal sequences.

**Example 5. Biological Activity.**

The above data demonstrate that the MCSF-223 and wild-type M-CSF mature forms are similar but not identical. An experimental program was designed to compare the biological activities of the proteins. The results of the program, as described below, demonstrate that the two materials are extremely similar, if not identical, in the biological properties tested. The in vitro specific activity of purified M-CSF from MCSF-223 cell lines and wild type cell lines was determined in the 32Dcfms assay (Table II). This assay determines the activity of M-CSF by measuring its ability to stimulate the proliferation of 32D murine myeloid cells which stably express the human M-CSF receptor (c-fms). Material was assayed in a full range dose response curve with a purified M-CSF standard as reference. The slopes of the curves for the various preparations were similar allowing the calculation of specific activity using the  $ED_{50}$  value. The in vitro specific activity of the purified MCSF-223 was similar to that of wild type M-CSF and within the variation of the assay. The  $ED_{50}$  of the standard has been defined as  $19.9 \pm 7.9$  ng, the specific activity has been defined as  $0.6 \pm 0.23 \times 10^5$  U/mg, and the slope as  $1.2 \pm 0.3$ . In addition, purified M-CSF from four MCSF-223 cell lines was tested in the mouse bone marrow assay. This assay determines the ability of M-CSF to stimulate proliferation of hematopoietic progenitor cells derived from murine bone marrow. All MCSF-223 samples were shown to have biological activity of  $> 0.5 \times 10^6$  U/mg which is similar to that obtained for wild type M-CSF.

TABLE II

M-CSF Bioassay Results for MCSF-223 and Wild-type M-CSF cell lines			
Cell line	Slope	ED <sub>50</sub> ng	S.A. U/mg
MCSF-223 Line H1	1.28	27.7	0.36x10 <sup>5</sup>
MCSF-223 Line D2	1.32	17.6	0.57x10 <sup>5</sup>
Wild-type M-CSF	1.31	27.3	0.37x10 <sup>5</sup>
Standard (Wild-type)	1.30	19.3	0.52x10 <sup>5</sup>

Legend: S.A., specific activity.

#### Example 6. Evaluation of Pharmacokinetics and Biologic Activity of MCSF-223.

The goal of these evaluations was to compare the pharmacokinetic and biologic activity of rhM-CSF produced by MCSF-223 cell lines to rhM-CSF produced by wild type M-CSF cell line. A two-tiered approach was used for this evaluation. The first step was a rat pharmacokinetic profile of rhM-CSF produced by four different MCSF-223 cell lines compared to control rhM-CSF. Two of the MCSF-223 cell line rhM-CSF samples used in the pharmacokinetic evaluation were then selected for a secondary evaluation of biologic activity in rats following intravenous and subcutaneous administration.

#### Pharmacokinetic Experimental Design.

Twenty female Sprague-Dawley rats were used for this evaluation. The rats were randomly divided into 5 groups of four rats each. The test groups received an intravenous

bolus injection of iodinated MCSF-223 protein produced by MCSF-223 cell lines. A control group received iodinated rhM-CSF produced by wild-type M-CSF. Rats were anesthetized by an IM injection of a mixture of Ketamine and Xylazine and dosed by tail vein injection with a mixture of  $^{125}\text{I}$  rhM-CSF and unlabelled rhM-CSF at a total dose of 50 ug/kg. Blood was taken at 0.5, 2, 5, 10, 15, 45, 90, 180 and 360 minutes post dosing and was allowed to clot at room temperature for 10 minutes. The samples were kept on ice for ten minutes and then centrifuged to separate the serum. Aliquots of serum (50ul) were counted for  $^{125}\text{I}$  in a gamma counter. Precipitable counts were determined in 20% TCA.

Serum rhM-CSF level versus time curves were constructed and fit by a mono-exponential equation of the form  $C_p = A \cdot e^{-kt}$  by a non-linear regression program. The half-life, volume of distribution and total body clearance for rhM-CSF in each animal were determined based on the slope and intercept of the best fit function.

The serum samples of selected rats ( $N=2/\text{Group}$ ) were also evaluated by SEC-HPLC. Serum (20ul) was injected directly onto a SEC-250 column (Biorad) and  $^{125}\text{I}$  was monitored using an on-line gamma detector (Berthold). The height of a single peak that eluted at the same apparent molecular weight as rhM-CSF was measured for each serum sample. The percent  $^{125}\text{I}$  rhM-CSF remaining was determined using the 0.5 minute peak height as 100%. Pharmacokinetic analysis was done as described above.

The serum half-life of precipitable radioactivity for rhM-CSF from MCSF-223 cell lines ranged from 54 minutes to 83 minutes. The half-life and clearance estimates of the MCSF-223 cell line variants were not statistically different from control values (Students T-test). The pharmacokinetic parameters of half-life, total body clearance, and volume of

distribution are summarized in Table III. The volume of distribution approximated serum volume and was similar for all rhM-CSF variants evaluated. The half-life values determined by SEC-HPLC were very similar to those obtained from precipitable serum radioactivity. Serum radioactivity eluted from the column as a single peak at the same retention time as rhM-CSF, suggesting that the precipitable counts in the serum represent intact rhM-CSF.

5

TABLE III

PHARMACOKINETIC SUMMARY OF MCSF-223 CELL LINES IN SPRAGUE DAWLEY RAT MEAN +/- SD				
CELL LINE	1/2-LIFE (MIN)	C <sub>MAX</sub>	TOTAL CLEARANCE (mL/MIN/KG)	VOLUME OF DISTRIBUTION (mL)
Line D2	54	877	0.75	19
	4	83	0.10	2
Line C10	53	975	0.69	17
	10	57	0.12	1
Line H1	69	916	0.55	18
	7	71	0.10	1
Line H8	83	909	0.47	17
	11	53	0.06	1
Control (wild-type)	70	873	0.60	18
	15	32	0.16	1

The lower number in each box represents the standard deviation

#### Example 7. Biologic Effects of MCSF-223 form rhM-CSF

Forty-eight female Sprague Dawley rats were divided randomly into eight groups of six animals each. Four groups received test article or vehicle control as a single subcutaneous injection of 2.5 mg/Kg. Four groups received test article or vehicle control as daily intravenous injections of 500 ug/kg/day for 5 consecutive days. The groups received MCSF-223 form of rhM-CSF, control rhM-CSF produced by wild-type M-CSF cell lines or vehicle control. The animals were anesthetized with isoflurane inhalant anesthetic and bled by cardiac puncture on days 0,2,4,6,8 and 15 onto EDTA. Hematological parameters, measured on a Baker 9000 hematology analyzer, included white blood cell count, red blood

cell count, platelet count, hemoglobin, hematocrit, and Weintrobe's constants. Differential cell counts (100 cells) were performed on Wrights-Geisma stained peripheral blood smears.

A decrease in platelet count has been the most reproducible observation following the administration of rhM-CSF to rats. The subcutaneous groups showed a maximum decrease on day 2 after dosing of between 30 and 40 percent below baseline. The kinetics of platelet decrease and recovery were similar for all subcutaneous rhM-CSF receiving groups (MCSF-223 cell lines and wild-type rhM-CSF cell lines). The intravenous groups consistently reached a low platelet count on day 4 at about 50 percent below baseline. The platelet decrease and rebound was similar for animals receiving MCSF-223 cell line rhM-CSF and control (wild-type cell line) rhM-CSF intravenously. No significant platelet effects were noted in the vehicle receiving groups.

A peripheral monocytosis is observed following the subcutaneous or intravenous administration of rhM-CSF to rats, but historically, this response has been more variable than the very consistent thrombocytopenia. Both dosing routes resulted in a variable peripheral monocytosis which reached a maximum on day 2 of the evaluation. No consistent changes were noted in the vehicle receiving group. MCSF-223 produced by the MCSF-223 cell line H1 showed the largest increase in total peripheral monocytes for both dosing routes.

Following intravenous administration to rats, the pharmacokinetic profile of rhmcsf produced by MCSF-223 cell lines were not statistically different from rhmcsf produced by wild-type M-CSF cell line. MCSF-223 produced by the MCSF-223 cell lines H1 and D2 produced platelet decreases following intravenous and subcutaneous administration to rats that were indistinguishable from that produced by rhM-CSF produced by wild-type M-CSF cell



ultrafiltration/diafiltration step has been incorporated prior to the initial QAE-Sephrose capture step. The diafiltration removes low molecular weight components and substantially enhances the capacity of the QAE-Sephrose column, allowing much smaller columns to be used. Additionally, ceramic Hydroxyapatite has been substitute for Hydroxyapatite Ultrigel.

5 The ceramic matrix has significantly higher capacity as well as more desirable handling properties. With the changes described above, the purificaion process for purification of the M-CSF from the 223 cell line now consists of ultrafiltration/diafiltration; then sequential chromatography on QAE-Sephrose, ceramic Hydroxyapatite and Phenyl-Toyopearl; and finally diafiltration to yield bulk drug substance in the appropriate buffer. This process

10 provides a robust purification scheme, readily able to handle the large amounts of M-CSF prodluuced by the 223 cell line and able consistently to yield a high purity product.

#### **Example 9. 250 L Bioreactor/Purification Scale-up Runs**

A 250 L bioreactor was used to produce six successful harvests from M-CSF cell lines. Each run has resulted in improvements with the % recovery approaching 95%.

15 Chromatographic purification was used to produce 1.3 runs. The major quantitative contaminant detected is the HMW-MCSF, at less than about 1%, a significant improvement over prior processes.

#### **Summary**

Extensive characterization studies of have shown that no significant differences exist

20 in biological characteristics and activity purified MCSF-223 protein compared to wild type M-CSF. The absence of high molecular weight proteoglycan species of M-CSF produced from MCSF-223 cell lines represents a significant advantage from both yield and protein

purification perspectives. We have shown that, using the MCSF-223 vector, volume productivity in production of M-CSF protein has been increased at least approximately 10-fold.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Dorner, Andrew  
Fritsch, Edward  
Steininger, Robert  
Bush, Lawrence

(ii) TITLE OF INVENTION: MCSF-223 Amino Acid Process

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Legal Affairs, Genetics Institute, Inc.  
(B) STREET: 87 CambridgePark Drive  
(C) CITY: Cambridge  
(D) STATE: MA  
(E) COUNTRY: USA  
(F) ZIP: 02140-2387

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 08/004,141  
(B) FILING DATE: 13-JAN-1993  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lazar, Steven R.  
(B) REGISTRATION NUMBER: 32,618  
(C) REFERENCE/DOCKET NUMBER: GI-5210

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-876-1170  
(B) TELEFAX: 617-876-5851

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Dorner, Andrew  
Fritsch, Edward  
Steininger, Robert  
Bush, Lawrence

(ii) TITLE OF INVENTION: MCSF-223 Amino Acid Process

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(A) ADDRESSEE: Legal Affairs, Genetics Institute, Inc.

(B) STREET: 87 CambridgePark Drive

(C) CITY: Cambridge

(D) STATE: MA

(E) COUNTRY: USA

(F) ZIP: 02140-2387

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 08/004,141

(B) FILING DATE: 13-JAN-1993

(C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lazar, Steven R.

(B) REGISTRATION NUMBER: 32,618

(C) REFERENCE/DOCKET NUMBER: GI-5210

## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-876-1170

(B) TELEFAX: 617-876-5851

Ala Lys Gln Arg Pro Pro Arg 250 255		
TCCCTCCCCC CCCCCTAACG TTA CTGGCCG AAGCCGCTTG GAATAAGGCC GGTGTGCGTT	2086	
TGTCTATATG TTATTTTCCA CCATATTGCC GTCTTTTGGC AATGTGAGGG CCCGGAAACC	2146	
TGGCCCTGTC TTCTTGACGA GCATTCTAG GGGTCTTCC CCTCTCGCCA AAGGAATGCA	2206	
AGGTCTGTTG AATGTCGTGA AGGAAGCAGT TCCTCTGGAA GCTTCTTGAA GACAAACAAC	2266	
GTCTGTAGCG ACCCTTTGCA GGCAGCGGAA CCCCCACCT GGCAGACAGGT GCCTCTGCGG	2326	
CCAAAAGCCA CGTGTATAAG ATACACCTGC AAAGGCGGCA CAACCCAGT GCCACGTTGT	2386	
GAGTTGGATA GTTGTGGAAA GAGTCAAATG GCTCTCTCA AGCGTATTCA ACAAGGGGCT	2446	
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CTA CCC TGG CCT CCG CTC AGG AAC GAG TTC AAG TAC TTC CAA AGA ATG Leu Pro Trp Pro Pro Leu Arg Asn Glu Phe Lys Tyr Phe Gln Arg Met 25 30 35	2715	
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AGA ATT AAT ATA GTT CTC AGT AGA GAA CTC AAA GAA CCA CCA CGA GGA Arg Ile Asn Ile Val Leu Ser Arg Glu Leu Lys Glu Pro Pro Arg Gly 75 80 85	2859	
GCT CAT TTT CTT GCC AAA AGT TTG GAT GAT GCC TTA AGA CTT ATT GAA Ala His Phe Leu Ala Lys Ser Leu Asp Asp Ala Leu Arg Leu Ile Glu 90 95 100	2907	
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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Gly Ser Leu Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser Ile Thr
          20          25          30
Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly His Leu
          35          40          45
Gln Ser Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser Cys Gln
          50          55          60
Ile Thr Phe Glu Phe Val Asp Gln Glu Gln Leu Lys Asp Pro Val Cys
          65          70          75          80
Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu Asp Thr
          85          90          95
Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu
          100          105          110
Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu
          115          120          125
Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln
          130          135          140
Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu
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Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala
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Gln Pro Leu Ala Pro Ser Met Ala Pro Val Ala Gly Leu Thr Trp Glu
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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 187 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Met Val Arg Pro Leu Asn Cys Ile Val Ala Val Ser Gln Asn Met Gly
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          35           40           45
Asn Leu Val Ile Met Gly Arg Lys Thr Trp Phe Ser Ile Pro Glu Lys
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Asn Arg Pro Leu Lys Asp Arg Ile Asn Ile Val Leu Ser Arg Glu Leu
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Lys Glu Pro Pro Arg Gly Ala His Phe Leu Ala Lys Ser Leu Asp Asp
          85           90           95
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 553 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..32
- (D) OTHER INFORMATION: /label= leader\_peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 33..553
- (D) OTHER INFORMATION: /note= "Mature MCSF peptide"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Thr Ala Pro Gly Ala Ala Gly Arg Cys Pro Pro Thr Thr Trp Leu  
 1 5 10 15  
 Gly Ser Leu Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser Ile Thr  
 20 25 30  
 Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly His Leu  
 35 40 45  
 Gln Ser Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser Cys Gln  
 50 55 60  
 Ile Thr Phe Glu Phe Val Asp Gln Glu Gln Leu Lys Asp Pro Val Cys  
 65 70 75 80  
 Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu Asp Thr  
 85 90 95  
 Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu  
 100 105 110  
 Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu  
 115 120 125  
 Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln  
 130 135 140  
 Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu  
 145 150 155 160  
 Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala  
 165 170 175  
 Glu Cys Ser Ser Gln Asp Val Val Thr Lys Pro Asp Cys Asn Cys Leu  
 180 185 190  
 Tyr Pro Lys Ala Ile Pro Ser Ser Asp Pro Ala Ser Val Ser Pro His  
 195 200 205  
 Gln Pro Leu Ala Pro Ser Met Ala Pro Val Ala Gly Leu Thr Trp Glu  
 210 215 220  
 Asp Ser Glu Gly Thr Glu Gly Ser Ser Leu Leu Pro Gly Glu Gln Pro  
 225 230 235 240  
 Leu His Thr Val Asp Pro Gly Ser Ala Lys Gln Arg Pro Pro Arg Ser  
 245 250 255  
 Thr Cys Gln Ser Phe Glu Pro Pro Glu Thr Pro Val Val Lys Asp Ser  
 260 265 270  
 Thr Ile Gly Gly Ser Pro Gln Pro Arg Pro Ser Val Gly Ala Phe Asn  
 275 280 285  
 Pro Gly Met Glu Asp Ile Leu Asp Ser Ala Met Gly Thr Asn Trp Val  
 290 295 300  
 Pro Glu Glu Ala Ser Gly Glu Ala Ser Glu Ile Pro Val Pro Gln Gly  
 305 310 315 320

Thr Glu Leu Ser Pro Ser Arg Pro Gly Gly Gly Ser Met Gln Thr Glu  
 325 330 335  
 Pro Ala Arg Pro Ser Asn Phe Leu Ser Ala Ser Ser Pro Leu Pro Ala  
 340 345 350  
 Ser Ala Lys Gly Gln Gln Pro Ala Asp Val Thr Gly Thr Ala Leu Pro  
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 Arg Val Gly Pro Val Arg Pro Thr Gly Gln Asp Trp Asn His Thr Pro  
 370 375 380  
 Gln Lys Thr Asp His Pro Ser Ala Leu Leu Arg Pro Pro Glu Pro Gly  
 385 390 395 400  
 Ser Pro Arg Ile Ser Ser Leu Arg Pro Gln Gly Leu Ser Asn Pro Ser  
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 465 470 475 480  
 Glu Arg Gln Ser Glu Gly Ser Ser Ser Pro Gln Leu Gln Glu Ser Val  
 485 490 495  
 Phe His Leu Leu Val Pro Ser Val Ile Leu Val Leu Leu Ala Val Gly  
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 Gly Leu Leu Phe Tyr Arg Trp Arg Arg Arg Ser His Gln Glu Pro Gln  
 515 520 525  
 Arg Ala Asp Ser Pro Leu Glu Gln Pro Glu Gly Ser Pro Leu Thr Gln  
 530 535 540  
 Asp Asp Arg Gln Val Glu Leu Pro Val  
 545 550

International Application No: PCT/

/

**MICROORGANISMS**Optional Sheet in connection with the microorganism referred to on page 10, line 5 of the description**A. IDENTIFICATION OF DEPOSIT**Further deposits are identified on an additional sheet ☐

Name of depositary institution \*

America Type Culture Collection

Address of depositary institution (including postal code and country) \*

12301 Parklawn Drive  
Rockville, MD 20852 United States of America

Date of deposit \*

December 11, 1992

Accession Number \*

ATCC 75378

**B. ADDITIONAL INDICATIONS** \* (leave blank if not applicable). This information is continued on a separate attached sheet ☐**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** \* (if the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS** \* (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later \* (Specify the general nature of the indications e.g., "Accession Number of Deposit")

**E.** ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau is

was

(Authorized Officer)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF  
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3  
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Genetics Institute, Inc.  
Attention: Andrew J. Dorner  
87 CambridgePark Drive  
Cambridge, MA 02140

Deposited on Behalf of: Genetics Institute, Inc.

Identification Reference by Depositor:

ATCC Designation

DNA Plasmid, p MEMCMat

75378

The deposit was accompanied by: ☐ a scientific description ☐ a proposed taxonomic description indicated above.

The deposit was received December 11, 1992 by this International Depository Authority and has been accepted.

AT YOUR REQUEST:

☒ We will inform you of requests for the strain for 30 years.

☐ strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested December 21, 1992. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Bobbie A. Brandon  
Bobbie A. Brandon, Head, ATCC Patent Depository

Date: January 4, 1993

cc: Steven R. Lazar

Form BP4/9

SUBSTITUTE SHEET (RULE 26)

International Application No: PCT/

**MICROORGANISMS**

Optional Sheet in connection with the microorganism referred to on page. 13, line 5-6 of the description \*

**A. IDENTIFICATION OF DEPOSIT \***Further deposits are identified on an additional sheet ☐ \*

Name of depository institution \*

America Type Culture Collection

Address of depository institution (including postal code and country) \*

12301 Parklawn Drive  
Rockville, MD 20852 United States of America

Date of deposit \*

February 19, 1993

Accession Number \*

ATCC CRL 11275

**B. ADDITIONAL INDICATIONS \*** (leave blank if not applicable). This information is continued on a separate attached sheet ☐**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE \*** (if the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS \*** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later \* (Specify the general nature of the indications e.g., "Accession Number of Deposit")

**E.** ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau is \*

was

(Authorized Officer)



# American Type Culture Collection

12301 Parklawn Drive • Rockville, MD 20852 USA • Telephone: (301) 231-5520 Telex: 896-055 ATCCNORTH • FAX: 301-770-2587

*create*  
ATCC  
CRL 11275  
Folden

## BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

### INTERNATIONAL FORM

#### RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Genetics Institute  
Attention: S. Robert Adamson  
87 CambridgePark Drive  
Cambridge, MA 02140

Deposited on Behalf of: Genetics Institute

Identification Reference by Depositor:

ATCC Designation

Chinese Hamster Ovary Cells, (7/1)CHO - H1 - 2.0

CRL 11275

The deposit was accompanied by: ☐ a scientific description ☐ a proposed taxonomic description indicated above.

The deposit was received February 19, 1993 by this International Depository Authority and has been accepted.

#### AT YOUR REQUEST:

☒ We will inform you of requests for the strain for 30 years.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested February 24, 1993. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Bobbie A. Brandon Date: February 25, 1993  
Bobbie A. Brandon, Head, ATCC Patent Depository

cc: Steven R. Lazar ✓

Form BP4/9

SUBSTITUTE SHEET (RULE 26)

We claim:

1. An isolated and purified DNA molecule consisting essentially of the DNA sequence of Sequence ID No. 1.
2. An isolated and purified DNA molecule according to claim 1, wherein the DNA molecule encodes the production of a 255 amino acid polypeptide, comprising a 32 amino acid leader sequence from M-CSF and a 223 amino acid polypeptide having M-CSF activity, said polypeptide lacking the carboxy-terminal propeptide sequence present in wild-type recombinant M-CSF polypeptide before intracellular processing when produced by mammalian cells.
3. An isolated and purified DNA molecule comprising the plasmid pMemcMAT.
4. An isolated and purified DNA molecule consisting essentially of a DNA sequence encoding the polypeptide comprised of the amino acid sequence of Sequence ID No. 2.

5. An isolated and purified DNA molecule according to claim 4, wherein the DNA molecule encodes the production of a 255 amino acid polypeptide, comprising a 32 amino acid leader sequence from M-CSF and a 223 amino acid polypeptide having M-CSF activity, said polypeptide lacking the carboxy-terminal propeptide sequence present in wild-type recombinant M-CSF polypeptide before intracellular processing when produced by mammalian cells.

6. A method of producing MCSF-223 protein comprising:

a) culturing in a suitable culture medium cells transformed with a DNA molecule according to claim 1; and

b) isolating and purifying said protein from said culture medium.

7. A method of producing MCSF-223 protein comprising:

a) culturing in a suitable culture medium cells transformed with the DNA molecule according to claim 3;

b) isolating and purifying said protein from said culture medium.

8. A mammalian cell line capable of producing MCSF-223 protein, said mammalian cell line comprising a DNA sequence according to claim 1.

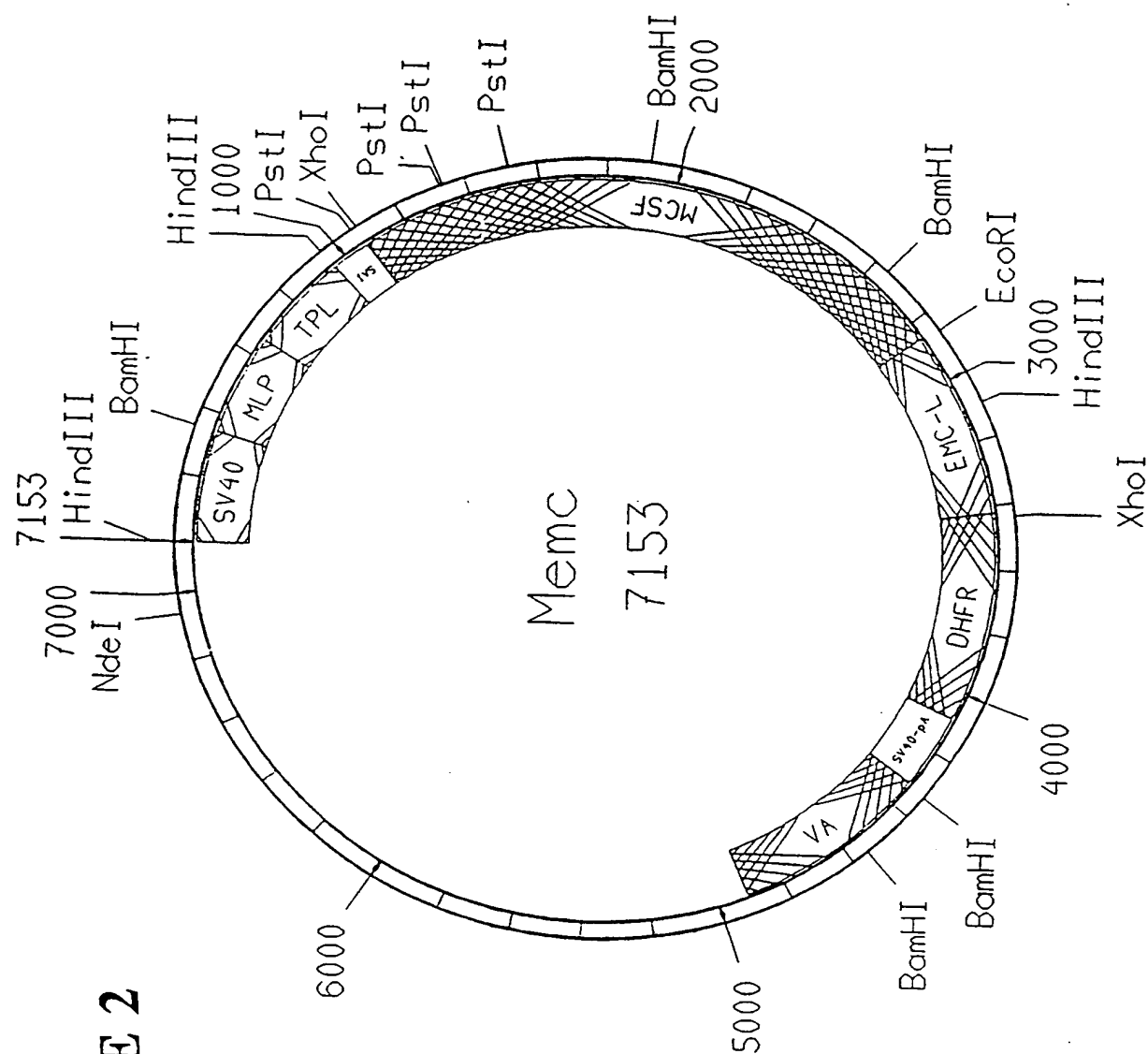
9. A mammalian cell line capable of producing MCSF-223 protein, said mammalian cell line comprising a DNA sequence according to claim 2.

10. A method of purifying MCSF-223 protein comprising the following steps in sequence:

- a) ultrafiltration/diafiltration;
- b) QAE- Sephrose chromatography;
- c) ceramic hydroxyapatite chromatography;
- d) Phenyl-Toyoperl chromatography; and
- e) diafiltration.

## FIGURE 1

MTAPGAAGRCPPTTWLGSLLLLVCLLASRSIT																									-32
E	E	V	S	E	Y	C	S	H	M	I	G	S	G	H	L	Q	S	L	Q	R	L	I	D	S	25
Q	M	E	T	S	C	Q	I	T	F	E	F	V	D	Q	E	Q	L	K	D	P	V	C	Y	L	50
K	K	A	F	L	L	V	Q	D	I	M	E	D	T	M	R	F	R	D	N	T	P	N	A	I	75
A	I	V	Q	L	Q	E	L	S	L	R	L	K	S	C	F	T	K	D	Y	E	E	H	D	K	100
A	C	V	R	T	F	Y	E	T	P	L	Q	L	L	E	K	V	K	N	V	F	N	E	T	K	125
N	L	L	D	K	D	W	N	I	F	S	K	N	C	N	N	S	F	A	E	C	S	S	Q	D	150
V	V	T	K	P	D	C	N	C	L	Y	P	K	A	I	P	S	S	D	P	A	S	V	S	P	175
H	Q	P	L	A	P	S	M	A	P	V	A	G	L	T	W	E	D	S	E	G	T	E	G	S	200
S	L	L	P	G	E	Q	P	L	H	T	V	D	P	G	S	A	K	Q	R	P	P	R	**^		223
S	T	C	Q	S	F	E	P	P	E	T	P	V	V	K	D	S	T	I	G	G	S	P	Q	P	248
R	P	S	V	G	A	F	N	P	G	M	E	D	I	L	D	S	A	M	G	T	N	W	V	P	273
E	E	A	<u>S</u>	<u>G</u>	<u>E</u>	<u>A</u>	S	E	I	P	V	P	Q	G	T	E	L	S	P	S	R	P	G	G	298
G	S	M	Q	T	E	P	A	R	P	S	N	F	L	S	A	S	S	P	L	P	A	S	A	K	323
G	Q	Q	P	A	D	V	T	G	T	A	L	P	R	V	G	P	V	R	P	T	G	Q	D	W	348
N	H	T	P	Q	K	T	D	H	P	S	A	L	L	R	O	P	P	E	P	G	S	P	R	I	373
S	S	L	R	P	Q	G	L	S	N	P	S	T	L	S	A	Q	P	Q	L	S	R	S	H	S	398
S	G	S	V	L	P	L	G	E	L	E	G	R	R	S	T	R	D	R	R	S	P	A	E	P	423
E	G	G	P	A	S	E	G	A	A	R	P	L	P	R	F	N	S	V	P	L	T	D	T	G	448
H	E	R	Q	S	E	G	S	S	S	P	Q	L	Q	E	S	V	F	H	L	L	V	P	S	V	473
I	L	V	L	L	A	V	G	G	L	L	F	Y	R	W	R	R	S	H	Q	E	P	Q	R		498
A	D	S	P	L	E	Q	P	E	G	S	P	L	T	Q	D	D	R	Q	V	E	L	P	V		522



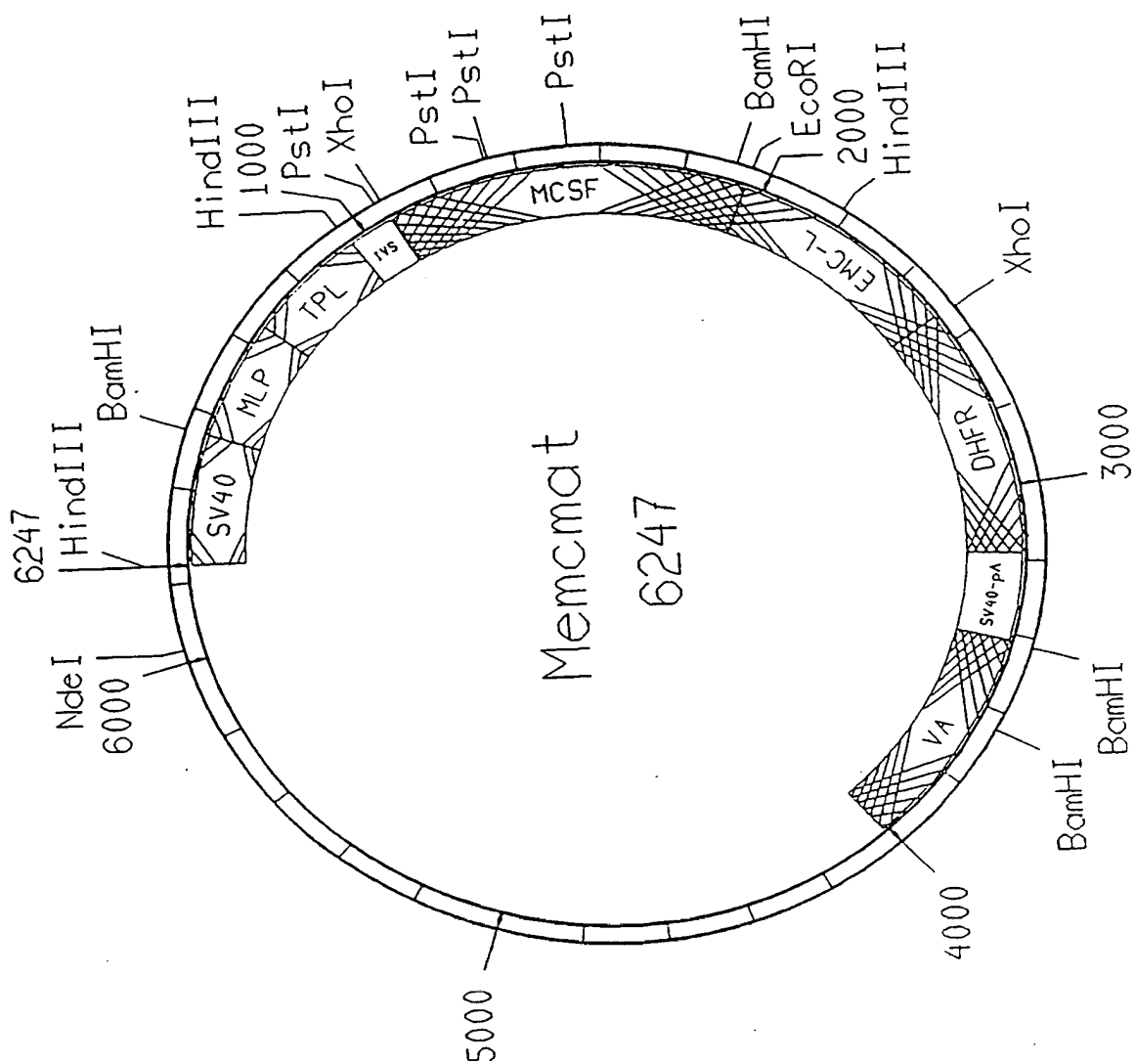


FIGURE 3

